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# Mapping of the region of the tick-borne encephalitis virus replicase adjacent to initiating substrate binding center

O.V. Morozova[1], A.A. Mustaev[3], N.A. Belyavskaya[2], E.F. Zaychikov[3], E.A. Kvetkova[2], Yu.I. Wolf[1] and A.G. Pletnev[1]

[1]*Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Academy of Sciences of the USSR, Novosibirsk-90, USSR,* [2]*Omsk Institute of Natural-Foci Infections, RSFSR Ministry of Health, Omsk-80, USSR* and [3]*Limnological Institute, Siberian Division of the Academy of Sciences of USSR, Irkutsk-33, USSR*

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Affinity labelling with aldehyde-containing analogs of initiation substrates of nuclear fraction of tick-borne encephalitis virus (TBEV) infected cells results in a labelling of a single polypeptide with a molecular mass of 68 kDa which was immunologically identified as TBEV NS3 protein. A single-hit hydroxylamine hydrolysis, using limited and long-term CNBr cleavages allowed one to identify $Lys^{1800}$ and/or $Lys^{1803}$ as the label attachment sites. These amino acid residues are situated in the proximity of the 'B'-site of NTP-binding motif of viral RNA replicase.

Flavivirus; Replicase; Affinity labelling; Active center

## 1. INTRODUCTION

In our recent work [1] the RNA-dependent RNA polymerase (replicase) of tick-borne encephalitis virus (TBEV) was identified in a nuclear fraction of infected cells using highly specific affinity labelling [2] according to the following scheme:

$$E\text{-}NH_2 \xrightarrow[(2)\ NaBH_4]{(1)\ OCH\text{-}R\text{-}pN_1} E\text{-}NH\text{-}CH_2\text{-}R\text{-}pN_1 \xrightarrow{ppp^*N_2}$$

$$E\text{-}NH\text{-}CH_2\text{-}pN_1\text{-}p^*N_2$$

where $E\text{-}NH_2$ is the enzyme, containing lysine residues, $OHC\text{-}R\text{-}pN_1$ is aldehyde-containing nucleotide derivate (affinity reagent), $ppp^*N_2$ is [$\alpha$-$^{32}$G]GTP (elongating substrate).

The radioactivity was registered in a single 69 kDa polypeptide which was immunologically proved to be NS3 viral protein. The labelling was achieved only when derivatives of 5′-nucleotide of viral plus-strand RNA (adenosine) were used as affinity reagents, and radioactive GTP (corresponding to the next nucleotide in the chain) as elongating substrate. The present work is aimed at mapping the labelled region of the NS3 protein with known amino acid sequence [3].

*Correspondence address:* A.G. Pletnev, Novosibirsk Institute of Bioorganic Chemistry, Lavrentiev prospect 8, 630090 Novosibirsk-90, USSR

## 2. MATERIALS AND METHODS

Nuclear fraction of TBEV-infected pig embryo kidney cells was prepared according to [1] 45 h after infection. 4-Formylphenyl ester of ATP (fp-ATP) was synthesized as described in [2].

### 2.1. *Affinity labelling of TBEV replicative complex*

Reaction mixture (20 $\mu$l) contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM $\beta$-mercaptoethanol, 50 mM NaCl, 14 $\mu$l of nuclear fraction of TBEV-infected cells and $5 \times 10^{-5}$ M fp-ATP incubated for 15 min at 30°C. Then 2 $\mu$l of 0.1 M $NaBH_4$ was added and the incubation continued for 30 min at 0°C. The mixture was supplemented with 2 $\mu$l of $10^{-5}$ M [$\alpha$-$^{32}$P]GTP (3000 Ci/mmol; 'Isotope', USSR) and incubated at 30°C for another 15 min. Then the mixture containing labelled NS3 protein was treated with 1% SDS (5 min, 100°C) and subjected to cleavages.

### 2.2. *Hydroxylamine cleavage*

10 $\mu$l of 2 M hydroxylamine/0.2 M potassium carbonate, pH 10, was added to 10 $\mu$l of the reaction mixture containing affinity labelled NS3 protein. The digestion was performed at 37°C for 1–2 h and stopped with 5 $\mu$l of 5% mercaptoethanol/0.5 M sodium acetate (pH 5)/50% glycerol. After heating at 100°C for 5 min, the products were separated by SDS gel electrophoresis in a gradient of 10–20% PAGE [4] followed by autoradiography.

### 2.3. *Cyanogen bromide cleavage*

(i) *Limited cleavage.* After affinity labelling and denaturation with SDS, the pH of the reaction mixture was adjusted to approximately 1.5 with 1–2 $\mu$l of 1 M HCl per 15 $\mu$l of the mixture, and 1 M CNBr was added to reach a 0.05 M concentration. Hydrolysis was carried out at room temperature. The reaction was stopped after 2, 4 or 6 min by mixing 4 $\mu$l aliquots with 1 $\mu$l of stop-mixture (5% mercaptoethanol/50% glycerol/0.5 M triethanolamine-HCl, pH 8.5).

(ii) *Long-term cleavage.* After SDS denaturation, the reaction mixture was treated with 1% dithiothreitol (25°C, 48 h) in order to reduce partially oxidized methionine residues. Then HCl (up to 0.05 M) and CNBr (up to 0.5 M) were added and the incubation proceeded for 48

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h. The probe was dissolved in a five-fold diluted stop-mixture and analyzed in 15-30% SDS-PAGE.

## 3. RESULTS

Among a number of ATP, ADP and AMP derivatives, used for labelling of TBEV replicase, the most effective affinity reagent appeared to be 4-formylphenyl $\gamma$-ester of ATP [1]. It bears an aldehyde group which selectively reacts with amino groups of lysine residues. The crosslink is made irreversible by borohydride reduction. The dependence of the affinity labelling of NS3 protein with fp-ATP on $NaBH_4$ treatment [1] gave proof for the attachment of the radioactive label to primary amino-groups.

To localize the labelled site on the polypeptide chain of NS3 we used a method based on limited cleavage of an affinity labelled protein at specific amino acid sequences [5]. It is well known [6] that at pH 10.0 hydroxylamine splits the amide bond between the neighboring Asn and Gly residues of polypeptide. There are 5 Asn-Gly sequences in the NS3 protein (Fig. 1). Depending on the position of a radioactive label different sets of radioactive single-hit peptides (peptides produced by a single hydroxylamine cleavage of the polypeptide chain) may appear. Theoretical electrophoretic patterns of radioactive peptides for different positions of the label are shown in Fig. 2 (lanes 3-6). The experimental pattern (Fig. 2, lanes 1-2) is similar to that of lane 4, proving that radioactive label is located between $Gly^{1732}$ and $Asn^{1824}$.

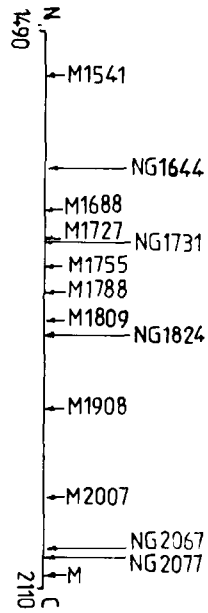


Fig. 1. Scheme of localization of CNBr and hydroxylamine cleavage sites on NS3 polypeptide chain. M, CNBr cleavage site; NG, hydroxylamine cleavage site; numbering starts from the beginning of TBEV polyprotein [3].

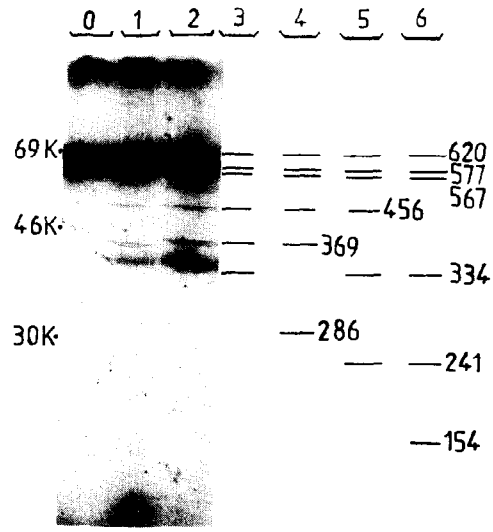


Fig. 2. Limited hydroxylamine cleavage of the affinity labelled TBEV replicase. (Lanes 1-3) Autoradiogram of the 10-20% gradient SDS-PAGE after electrophoretic separation of products of 0 h (1), 1 h (2) and 2 h (3) $NH_2OH$ treatment. (Lanes 4-7) Theoretical patterns of radioactive single-hit products for the label position between $Asn^{1731}$-$Asn^{1824}$ (4), $Asn^{1824}$-$Asn^{2067}$ (5), $Asn^{1644}$-$Asn^{1731}$ (6), $Ser^{1490}$-$Asn^{1644}$ (7) of NS3 protein ($Ser^{1490}$ is N-terminal). M, molecular weight markers. Polypeptide lengths are expressed as numbers of amino acid residues.

The shortest single-hit radioactive peptide, the product of limited CNBr cleavage at methionines, has a molecular mass of 34 kDa (Fig. 3, lanes 4-8), which makes a half of that for NS3 (69 kDa). Hence, the label must be located somewhere in the middle of the NS3 polypeptide chain. Unfortunately, the patterns of radioactive bands were very complicated suggesting three possible locations for the site of labelling:

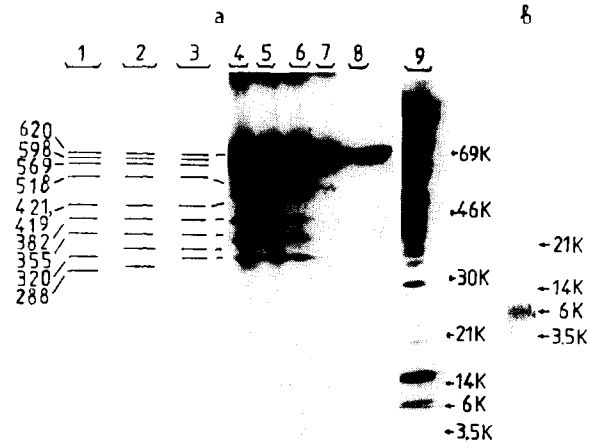


Fig. 3. Cyanogen bromide cleavage of TBEV replicase. (A) Limited cleavage. (Lanes 1-3) Putative patterns for the label attached between: $Met^{1755}$-$Met^{1778}$ (1), $Met^{1809}$-$Met^{1908}$ (2), $Met^{1778}$-$Met^{1809}$ (3). (Lanes 4-8) Autoradiogram of the 15-30% gradient SDS-PAGE after separation of products of CNBr treatment 8, 6, 4, 2 and 0 min (Lane 9) 40 min cleavage. (B) 48 h cleavage (15-30% SDS-PAGE). M, molecular weight markers.

$Met^{1755}$-$Met^{1778}$, $Met^{1778}$-$Met^{1809}$, and $Met^{1809}$-$Met^{1908}$ (Fig. 3, lanes 1-3). The first one, however, can be ruled out as containing no lysine residues.

A longer treatment of the labeled replicase with CNBr gives rise to a 6 kDa peptide (Fig. 3A, lane 9) which becomes the only product of the 48 h hydrolysis (Fig. 3B). Its weight is too small for the $Met^{1809}$-$Met^{1908}$ fragment ($M_r$ ~1.2 kDa of the attached dinucleotide should be taken into account), but fits well in that of the $Met^{1778}$-$Met^{1809}$ stretch of 31 amino acid residues. The latter contains two possible targets for fp-ATP; $Lys^{1800}$ and $Lys^{1803}$.

## 4. DISCUSSION

The data presented above give proof that $Lys^{1800}$ and/or $Lys^{1803}$ of TBEV NS3 protein are attachment sites for affinity labelling with fp-ATP. In principle, any of the three histidine residues contained within the complete CNBr fragment could be cross-linked [11]. However, aldehyde-histidine adducts should be stable enough to survive the electrophoresis, in contrast to the respective lysine-aldehyde derivatives, which are unstable unless stabilized by reduction [12]. Therefore, we favour $Lys^{1800}$ or $Lys^{1803}$ as the most plausible targets of linking. In accordance with the scheme of labelling these residues must be situated in or in very close proximity (not more than 1.3 nm apart) to the TBEV replicase initiation substrate binding site.

In our previous studies we have mapped affinity labelling sites for a number of bacterial and eukaryotic RNA polymerases [5,7,8]. All of them belonged to highly conserved domains which actually means that the structures forming RNA polymerases initiating substrate binding sites are evolutionary stable. This phenomenon agrees with the known evolutionary stability of functionally important structures in other proteins. Hence, it was interesting to compare aligned sequences for NS3 protein of TBEV and other flaviviruses [3,9]. $Lys^{1800}$ and $Lys^{1803}$ residues are evidently not conserved, and therefore seem to be functionally not essential, but they are surrounded by conserved sequences. NS3 proteins of flaviviruses contain an NTP-binding motif found in a vast group of (+)-strand RNA virus proteins [9]. The 'B'-site of this motif, surrounded by classical secondary structure (<$\beta$-strand>-'B'-<$\alpha$-helix>, as predicted by Garnier method [11]) is situated 21 amino acid residues to the left from $Lys^{1800}$. There are no other lysine residues nearby to $Lys^{1800/1803}$, so fp-ATP may be forced to react with more distant $Lys^{1800/1803}$ residues, situated, however, not far from the binding site. The use of affinity reagents bearing reactive groups with other chemical specificities and shorter 'arms' may give more definite information.

## REFERENCES

[1] Morozova, O.V., Belyavskaya, N.A., Zaychikov, E.F., Kvetkova, E.A., Mustaev, A.A. and Pletnev, A.G. (1990) Bioorgn. Khimia 6, 492-500.
[2] Grachev, M.A., Kolocheva, T.I., Lukhtanov, E.A. and Mustaev, A.A. (1987) Eur. J. Biochem. 163, 113-121.
[3] Pletnev, A.G., Yamshikov, V.F. and Blinov, V.M. (1990) Virology 174, 250-263.
[4] Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
[5] Grachev, M.A., Lukhtanov, E.A., Mustaev, A.A., Zaychikov, E.F., Abdukayumov, M.N., Rabinov, I.V., Richter, V.I., Skoblov, Yu.S. and Chistyakov, P.G. (1989) Eur. J. Biochem. 180, 577-585.
[6] Bornstein, P. and Balian, D. (1977) Methods Enzymol. 47, 132-145.
[7] Grachev, M.A., Mustaev, A.A., Zaychikov, E.F., Lindner, A.J. and Hartmann, G.R. (1989) FEBS Lett. 258, 317-332.
[8] Zaychikov, E.F., Mustaev, A.A., Glaser, S.J., Thomm, M., Grachev, M.A. and Hartmann, G.R. (1990) System. Appl. Microbiol. 13, 248-254.
[9] Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. and Blinov, V.M. (1989) Nucleic Acids Res. 17, 4713-4730.
[10] Garnier, J., Osguthorpe, D.J. and Robson, E. (1978) J. Mol. Biol. 120, 97-120.
[11] Ebralidse, K.K., Grachev, S.A. and Mirzabekov, A.D. (1988) Nature 331, 365-367.
[12] Guschin, D.Y., Nacheva, G.A., Preobrazhenskaya, O.V., Karpov, V.L., Ebralidse, K.K. and Mirzabekov, A.D. (1988) Proc. Acad. Sci. USSR (Moscow) 303, 1496-1500.